

02-25-02

JC07 Rec'd PCT/PTO 21 FEB 2002

10069689 .0819 PCT

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FORM PTO-1390
(REV. 9-2001)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

13189-PCT-US

U.S. APPLICATION NO. (If known, see 37 CFR 1.5

10/069689

INTERNATIONAL APPLICATION NO.
PCT/EP00/08270

INTERNATIONAL FILING DATE
22 August 2000

PRIORITY DATE CLAIMED
22 August 1999

TITLE OF INVENTION

IMPROVED METHOD FOR NUCLEOTIDE DETECTION AND DEVICES USED THEREIN

APPLICANT(S) FOR DO/EO/US

THUNNISSEN, Fredericus Bernardus Josephus Maria and HARDY, Katinka

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☒ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☐ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☒ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
14. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☒ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information: Markup of Amendments; Int'l Search Report; Notification of Transmittal of IPER; IPER.

U.S. APPLICATION NO. 10/069689 <small>(See 37 CFR 1.5)</small>	INTERNATIONAL APPLICATION NO. PCT/EP00/08270	ATTORNEY'S DOCKET NUMBER 13189-PCT-US
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21. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1040.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$890.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$740.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$710.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00 ENTER APPROPRIATE BASIC FEE AMOUNT =	CALCULATIONS PTO USE ONLY <table style="width: 100%;"> <tr> <td style="width: 60%;">\$ 890.00</td> <td style="width: 40%;"></td> </tr> </table>	\$ 890.00																					
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Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	<table style="width: 100%;"> <tr> <td style="width: 60%;">\$</td> <td style="width: 40%;"></td> </tr> </table>	\$																					
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<table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 20%;">CLAIMS</th> <th style="width: 20%;">NUMBER FILED</th> <th style="width: 20%;">NUMBER EXTRA</th> <th style="width: 20%;">RATE</th> <th style="width: 20%;">\$</th> </tr> </thead> <tbody> <tr> <td>Total claims</td> <td>14 - 20 =</td> <td>0</td> <td>x \$18.00</td> <td>\$ 0</td> </tr> <tr> <td>Independent claims</td> <td>2 - 3 =</td> <td>0</td> <td>x \$84.00</td> <td>\$</td> </tr> <tr> <td colspan="4">MULTIPLE DEPENDENT CLAIM(S) (if applicable) + \$280.00</td> <td>\$</td> </tr> </tbody> </table>	CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$	Total claims	14 - 20 =	0	x \$18.00	\$ 0	Independent claims	2 - 3 =	0	x \$84.00	\$	MULTIPLE DEPENDENT CLAIM(S) (if applicable) + \$280.00				\$	<table style="width: 100%;"> <tr> <td style="width: 60%;">\$</td> <td style="width: 40%;"></td> </tr> </table>	\$	
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<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.	<table style="width: 100%;"> <tr> <td style="width: 60%;">\$</td> <td style="width: 40%;"></td> </tr> </table>	\$																					
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Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +	<table style="width: 100%;"> <tr> <td style="width: 60%;">\$</td> <td style="width: 40%;"></td> </tr> </table>	\$																					
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a. ☒ A check in the amount of \$ 445.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees.
 A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any
 overpayment to Deposit Account No. 11-0171. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card
 information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR
 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Kalow & Springut LLP
 488 Madison Avenue, 19th Floor
 New York, NY 10022

SIGNATURE
 Tor E. Smeland
 NAME
43,131
 REGISTRATION NUMBER

Application Data Sheet**Application Information**

Application Type::	Regular
Subject Matter::	Utility
Title Line One::	Improved Method for Nucleotide Detection and
Title Line Two::	Devices used Therein
Attorney Docket Number::	13189-PCT-US
Request for Early Publication?::	No
Request for Non-Publication?::	No
Suggested Drawing Figure::	
Total Drawing Sheets::	10
Small Entity?::	Yes

Applicant Information

Applicant Authority type::	Inventor
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Country of Residence::	The Netherlands
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Country of mailing address:: Belgium

Correspondence Information

Correspondence Customer Number:: 23719

Representative Information

Representative Customer Number::	23719
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Foreign Priority Information

Country::	Application number::	Filing Date::	Priority Claimed::
EPO	PCT/EP00/08270	08/22/00	Yes
EP	99202714.4	08/22/99	Yes

Assignee Information

Assignee name:: Dot Diagnostics B.V.
Street of mailing address Line 1:: Tempelstraat 12A
City of mailing address:: Beuningen
Postal or Zip Code of mailing address:: 6641 KV
Country of mailing address:: Netherlands

1006968107069689

JC13 Rec'd PCT/PTO 21 FEB 2002

13189-PCT-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Thunnissen et al. Examiner: To be assigned
Serial No.: To be assigned Art Unit: To be assigned
Filed: Herewith
Title: Improved Method for Nucleotide Detection and Devices Used Therein

Certificate of Mailing Under 37 C.F.R. 1.10

I hereby declare that this correspondence is being deposited with the United States Postal Service via Express Mail Label No. EVO35775267US in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C.

Date: 2/21/02 Name: J. Colwell

Kalow & Springut LLP
488 Madison Avenue
New York, New York 10022

February 21, 2002

Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Dear Sir:

Prior to examination on the merits of this national stage application under the Patent Cooperation Treaty, please enter the following amendments in the above-identified application.

AMENDMENTS

IN THE SPECIFICATION:

On page 1 after the title and before "Field of Invention," please insert the following paragraph:

Cross-Reference to Related Applications

This application is a national stage filing under the Patent Cooperation Treaty (PCT) for PCT international application number PCT/EP00/08270 filed on 22 August 2000, published under

Applicant: Thunnissen et al.
Serial No.: Not yet assigned
Docket 13189-PCT-US
Preliminary Amendment – February 21, 2002
Page 2 of 6

PCT Article 21(2) in English as WO 01/18241 on 15 March 2001. Both the international application and this national stage application claim the benefit of the filing date of EP 99202714.4 filed 22 August 1999.

IN THE CLAIMS

Please amend claims 1-9 to read as follows:

1. (Amended) A method of detecting the presence of a nucleotide sequence within a double-stranded DNA in a sample, said method comprising the following steps:

a. coating a solid glass support with a first layer of biotinylated serum albumin in an amount to create sufficient binding sites for capture probes, drying said first layer to form a first dried layer and incubating said first dried layer with a second layer of streptavidin having sufficient density to perform efficient microarray analysis;

b. digesting a double-stranded DNA with an exonuclease to convert said double-stranded DNA to single-stranded DNA;

c. capturing a first nucleic acid probe adapted by biotin to said coated solid glass support;

d. hybridizing (i) the single-stranded DNA with the first nucleic acid probe, and (ii) one or more second nucleic acid probes, wherein said second nucleic acid probe is labeled with a detectable moiety and can hybridize with the single-stranded DNA adjacent the hybridized first nucleic probe;

e. ligating the hybridized first and second nucleic acid probes in case of a perfect match;

f. denaturing the ligated first and second nucleic acid probes from the single-stranded DNA to which they were hybridized;

g. removing labeled probes that are not covalently bound and single stranded DNA;

and

h. detecting a detectable moiety that has been ligated to said first nucleic acid probe indicating the presence of the nucleotide sequence within the double-stranded DNA in the sample;
 wherein steps c.-h. are performed by microarray technique.

2. (Amended) The method of claim 1, wherein said one or more second nucleic acid probes comprise a mixture of partly randomized probes to allow detection of mutations without knowing the site and type of mutation beforehand.

3. (Amended) The method according to claim 1, wherein said solid glass support is made of Starfrost glass.

4. (Amended) The method of claim 1, wherein the one or more first nucleic acid probes are placed on said solid glass support by light-directed oligonucleotide synthesis.

5. (Amended) The method of claim 1, wherein the detectable moiety on the second nucleic acid probe is digoxigenin, and the detecting step is performed by binding the digoxigenin with anti-digoxigenin antibody fragments.

6. (Amended) A device suitable for carrying out the detection method of claim 1, which comprises a solid glass support having a first layer of biotinylated serum albumin and a second layer comprising streptavidin, said layers having sufficient density to perform efficient microarray analysis.

7. (Amended) The device according to claim 8, wherein said solid glass support is

made of Starfrost glass.

8. (Amended) A kit comprising:

- a. a device suitable for carrying out the detection method of claim 1;
- b. a first nucleic acid probe which binds to target DNA and which is adapted with a capture moiety; and
- c. a second nucleic acid probe which binds to target DNA adjacent the first probe and which is labeled with a detectable moiety.

9. (Amended) A method for organizing microarray analysis on a solid support for rapid visual detection of abnormalities which comprises arranging a duplicate set of probes where a first series of arrays are for wild-type mutation order and a second series of arrays are for the classical sequencing order.

Please add the following new claims 10-14:

10. (New) The method of claim 1, wherein prior to drying said first layer in step a, Parafilm, Parafilm covered by a weight, or a surfactant is employed to enhance the distribution of said first layer.

11. (New) The device of claim 6, wherein the solid glass support is obtained by prior to drying said first layer in step a, Parafilm, Parafilm covered by a weight or a surfactant is employed to enhance the distribution of said first layer.

Applicant: Thunnissen et al.
Serial No.: Not yet assigned
Docket 13189-PCT-US
Preliminary Amendment – February 21, 2002
Page 5 of 6

12. (New) The kit of claim 8, further comprising an exonuclease.
13. (New) The kit of claim 8, further comprising a ligase.
14. (New) The kit of claim 8, further comprising an exonuclease and a ligase.

REMARKS

Entry of this preliminary amendment is respectfully requested. Pursuant to 37 CFR §1.121, a marked up version of the amendment to the specification and to the amended claims is submitted herewith.

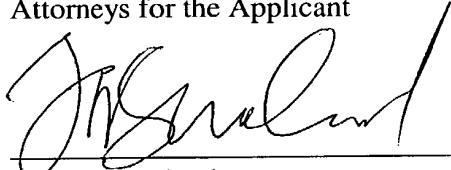
The claim amendments reflect (a) Article 19 amendments under the PCT filed during pendency of the PCT application of which this application is a national stage filing, and (b) changes to conform the claims to U.S. practice. If the Examiner would like separate markups of the claims representing Article 19 amendments to the claims as filed in the PCT application and markups of the claims representing amendments to the claims to conform to U.S. practice, the Examiner is invited to request such markups from Applicants. The claim amendments and the new claims add no new matter.

This is a PCT national stage filing under 35 USC §371, claiming a right of priority to an earlier filed foreign application EP 99202714.4 filed 22 August 1999, in accordance with 35 USC §363 and 35 USC §365. The amendment to the specification identifies the present application as a national stage filing in the U.S. under the PCT (for PCT/EP/00/08270 filed 22 August 2000) and also claims priority to EP 99202714.4 filed 22 August 1999.

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Serial No.: Not yet assigned
Docket 13189-PCT-US
Preliminary Amendment – February 21, 2002
Page 6 of 6

Applicants submit no fee is required in connection with the filing of this Preliminary Amendment. If any fee is deemed necessary, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 11-0171.

Respectfully submitted,
Attorneys for the Applicant



by Tor Smeland
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Kalow & Springut LLP
488 Madison Ave.
New York, New York 10022

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Facsimile: (212) 813-9600

10069689 10/069689

JC13 Rec'd PCT/PTC 21 FEB 2002

13189-PCT-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Thunnissen et al. Examiner: To be assigned
Serial No.: To be assigned Art Unit: To be assigned
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Title: Improved Method for Nucleotide Detection and Devices Used Therein

Certificate of Mailing Under 37 C.F.R. 1.10

I hereby declare that this correspondence is being deposited with the United States Postal Service via Express Mail Label No. EVO357752674 in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C.
Date: 2/21/02 Name: J. Colwell

Kalow & Springut LLP
488 Madison Avenue
New York, New York 10022

February 21, 2002

Commissioner for Patents
Washington, D.C. 20231

MARKUP OF AMENDMENTS PURSUANT TO 37 CFR 1.121

IN THE SPECIFICATION:

On page 1 after the title and before "Field of Invention," please insert the following paragraph:

Cross-Reference to Related Applications

This application is a national stage filing under the Patent Cooperation Treaty (PCT) for PCT international application number PCT/EP00/08270 filed on 22 August 2000, published under PCT Article 21(2) in English as WO 01/18241 on 15 March 2001. Both the international application and this national stage application claim the benefit of the filing date of EP 99202714.4 filed 22 August 1999.

Applicant: Thunnissen et al.
Serial No.: Not yet assigned
Docket 13189-PCT-US
Markup of Amendments – February 21, 2002
Page 2 of 6

IN THE CLAIMS:

Please amend claims 1-9 to read as follows:

1. (Amended) A method of detecting the presence of a nucleotide sequence within a double-stranded DNA in a sample, said method comprising the following steps:

a. coating a solid glass support with a first layer of biotinylated serum albumin[, and] in an amount to create sufficient binding sites for [the] capture probes, drying said first layer to form a first dried layer and incubating said first dried layer with a second layer of streptavidin having sufficient density to perform efficient microarray analysis;

b. digesting [the] a double-stranded DNA with an exonuclease to convert said double-stranded DNA to single-stranded DNA[, derived from a mixture of target cells and other cells, to a single-stranded DNA];

c. capturing a first nucleic acid probe adapted by biotin to said coated solid glass support [defined in step a];

d. hybridizing (i) the single-stranded DNA with the first nucleic acid probe, and (ii) [a] one or more second nucleic acid probes, wherein said second nucleic acid probe is labeled with a detectable moiety [which] and can hybridize with the single-stranded DNA adjacent the hybridized first nucleic probe;

Applicant: Thunnissen et al.
 Serial No.: Not yet assigned
 Docket 13189-PCT-US
 Markup of Amendments – February 21, 2002
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e. ligating the hybridized first and second nucleic acid probes in case of a perfect match [only];

f. denaturing the ligated first and second nucleic acid probes from the [hybridized] single-stranded DNA to which they were hybridized;

g. removing [non-covalently bound] labeled probes that are not covalently bound and single stranded DNA; and

h. detecting a [captured] detectable moiety that has been ligated to said first nucleic acid probe indicating the presence of the nucleotide sequence within the double-stranded DNA in the sample;

[characterized] wherein [in that] steps c.-h. are performed by microarray technique.

2. (Amended) The method of claim 1, wherein said one or more second nucleic acid probes comprise a mixture of partly randomized probes [step d (ii) is adapted with the use of a mixture of partly randomized probes] to allow detection of mutations without knowing the site and type of mutation beforehand.

3. (Amended) The method according to claim 1 or claim 2, wherein said solid glass support is made of [glass, preferably] Starfrost glass.

4. (Amended) The method of [any one of] claim[s] 1 [to 3], wherein the one or more first nucleic acid probes are placed [printed on said solid glass support or are built] on said solid glass support by light-directed oligonucleotide synthesis.

Applicant: Thunnissen et al.
Serial No.: Not yet assigned
Docket 13189-PCT-US
Markup of Amendments – February 21, 2002
Page 4 of 6

5. (Amended) The method of [any one of] claim[s] 1 [to 4], wherein the detectable moiety on the second nucleic acid probe is digoxigenin, and the detecting step is performed by binding the digoxigenin with anti-digoxigenin antibody fragments.

6. (Amended) A device suitable for carrying out the detection method of claim 1 [as claimed in any one of the preceding claims], which comprises a solid glass support having a [coating which is obtainable by the method comprising coating said solid glass support with a] first layer of biotinylated serum albumin and a [in an amount to create sufficient binding sites for the capture probes, drying said first layer, and incubating said first dried layer with a] second layer comprising [comprising a first layer of] streptavidin, said layers having sufficient density to perform efficient microarray analysis.

7. (Amended) The device according to claim 8, wherein [the] said solid glass support is made of [glass, preferably] Starfrost glass.

8. (Amended) A kit comprising:

a. a device suitable for carrying out the detection method [according to the present invention as claimed in any one] of claim[s] 1 [to 7];

[b. optionally an exonuclease;]

[c] b. a first nucleic acid probe which binds to target DNA and which is adapted with a capture moiety; and

Applicant: Thunnissen et al.
Serial No.: Not yet assigned
Docket 13189-PCT-US
Markup of Amendments – February 21, 2002
Page 5 of 6

[d] c. a second nucleic acid probe which binds to target DNA adjacent the first probe and which is labeled with a detectable moiety[; and].

[e. optionally a ligase.]

9. (Amended) A method for organizing microarray analysis on a solid support for rapid visual detection of abnormalities which comprises arranging a duplicate set of probes where a [the] first series of arrays are for [the] wild-type mutation order and a [the] second series of arrays are for the classical sequencing order.

Please add the following new claims 10-14:

10. (New) The method of claim 1, wherein prior to [the] drying said first layer in step a, Parafilm, Parafilm covered by a weight, or [parafilm, preferably covered by a weight, or] a surfactant is employed [added] to enhance the distribution of said first layer.

11. (New) The device of claim 6, wherein the solid glass support is obtained [obtainable] by [the method in which] prior to [the] drying said first layer in step a, Parafilm, Parafilm covered by a weight[, parafilm, preferably covered by a weight,] or a surfactant is employed [added] to enhance the distribution of said first layer.

12. (New) The kit of claim 8, further comprising an exonuclease.

13. (New) The kit of claim 8, further comprising a ligase.

Applicant: Thunnissen et al.
Serial No.: Not yet assigned
Docket 13189-PCT-US
Markup of Amendments – February 21, 2002
Page 6 of 6

14. (New) The kit of claim 8, further comprising an exonuclease and a ligase.

Respectfully submitted,
Attorneys for the Applicant

A handwritten signature in black ink, appearing to read 'Tor Smeland', is written over a horizontal line.

by Tor Smeland
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10/pts

Improved method for nucleotide detection and devices used therein

Field of the Invention

The present invention is in the field of molecular biology and diagnostics, and
5 relates in particular to an improved method of detecting the presence of nucleotide sequences, e.g. point mutations, within a double-stranded DNA. The method is useful, for example, in the early detection of lung and colon cancer.

Background of the Invention

10 Cancer is the second overall leading cause of death, after ischemic heart disease, in the United States and Western Europe and despite recent advances in its treatment, there is, for most cancer types, no miracle cure on the horizon. Cancer causes approximately 25 % of all deaths. The incidence continues to rise, probably reflecting the increasing average age of the population. The key to survival is early diagnosis and
15 treatment.

Lung cancer has a high incidence and mortality. Early detection programs with conventional methods such as X-ray and sputum cytology have failed to improve mortality. Lung carcinomas are now considered a genetic disease. Many regions in the genome have been thought to contain candidate genes related to the development of lung cancer.
20 The mutations in p53 and Kirsten ras (K-ras) genes are the best characterized in lung cancer and are thought to occur late in the development of lung cancer. Therefore, new approaches that use genetic alterations such as K-ras as potential biomarkers may be beneficial for early detection of lung cancer (Somers V.A.M.C., Thesis Maastricht University (1998), Netherlands).

25 Point mutations in the human genome play a central role in tumorigenesis (Bishop M.H., *Science* (1987) 235:305-311). Several methods for detection of known point mutations have been disclosed, which to a variable extent are time-consuming, technically complex, or hazardous due to the use of radioactive materials. See, e.g., Caskey C.T., *Science* (1987) 236:1223-1229; Landegren U., *et al.*, *Science* (1988) 242:229-237; and
30 Sommer S.S., *et al.*, *BioTechniques* (1992) 12:82-87.

Holloway B., *et al.*, *Nucl. Acids Res.* (1993) 21:3905-3906, disclose an exonuclease-amplification coupled capture technique ("EXACCT") which improved detection of PCR product.

Based on this technique, Murtagh and Thunnissen developed a simple, highly
35 specific non-radioactive microtiter plate format for detection of PCR products offering a

high sensitivity towards the detection of known point mutations, which was illustrated for the detection of human K-ras oncogene. See, e.g., Thunnissen F.B.J.M., Murtagh Jr., J.J., Somers V.A.M.C., *et al.*, *Lung* (1994) 11 (Suppl. 1):19, U.S. Patent No. 5,518,901, U.S. Patent No. 5,744,306, Somers V.A.M.C., *et al.*, *Nucl. Acids Res.* (1994) 22:4840-4841, 5 and Somers V.A.M.C., Thesis *ibid.*

Briefly, this method is based on the following principle: after exonuclease digestion, polymerase chain reaction fragments are determined by simultaneous hybridization with a capture probe and a detection probe complementary to sequences near the 3' end of the antisense fragment. The capture probe bears a biotin residue and 10 the detection probe digoxigenin. After hybridization, the PCR product hybrids are captured in streptavidin-coated microtiter plates and detected with labeled anti-digoxigenin antibody. For the detection of known point mutations this procedure was extended by using after the capture step the ligation of a mutation-specific capture probe with adjacent detection probe ("Point-EXACCT").

15 Essential in the Point-EXACCT technique is that only molecules will be detected by this format which have been hybridized with two probes and subsequently ligated, resulting in a very high degree of specificity. In addition, it has been found that Point-EXACCT requires considerably less time and effort as compared to other techniques used for the detection of known point mutations. The method can be easily automated, 20 permitting rapid screening of tissue banks with multiple probes to individual base substitutions, deletions or additions.

Various attempts have been made to further improve and optimize Point-EXACCT and other point mutation detection methods. Somers V.A.M.C., *et al.*, *Biochimica et Biophysica Acta* (1998) 1379:42-52, disclose an improvement of solution hybridization 25 after exonuclease pretreatment of amplification products for fluorescent cycle sequencing and point mutation detection. Digestion of a double-stranded amplification product to single strands by T7 gene 6 exonuclease increases hybridization efficiency and confers increased sensitivity and specificity of detection. The use of single-stranded amplification products gave by far the best results and is therefore almost required, especially in 30 particular cases. A prominent example is that with this approach DNA of one mutated cell can be detected in a DNA background of 15,000 wild type cells.

Point mutations in the K-ras oncogene are one of the most common genetic alterations involved in various types of human cancer. In lung cancer, K-ras mutations occur predominantly in codon 12. The frequency of those alterations varies within different

histological subtypes. K-ras point mutations are found in approximately 15-56% of the adenocarcinomas and to a lesser extent in other types of non-small cell lung carcinomas (NSCLC). K-ras point mutations at codon 12 are found to occur early during lung cancer development. See Somers V.A.M.C., Thesis *ibid.*, and references mentioned therein.

- 5 Somers V.A.M.C., *et al.*, *Clinical Chemistry* (1998) compared the detection of K-ras point mutations by Point-EXACCT with direct sequencing of double and single-stranded amplification products. Point-EXACCT showed clear advantages as compared to previously described highly sensitive methods.

In conclusion, the Point-EXACCT has been designed for analysis of single
10 base substitutions, where the exact place of said substitutions in the nucleotide sequence of the gene to be analyzed is known beforehand. Validation of the method for the detection of known point mutations in a large group of patients with NSCLC has confirmed its high sensitivity. Importantly, with this technique a relatively low amount of target cells is required before a signal is obtained. The ligation step is crucial. The whole procedure is
15 laborious, and therefore there is a clear need for an accurate and more efficient method.

Recently, 'chip' or 'microarray' technology has been developed, which is disclosed in, e.g., U.S. Patent No. 5,445,934. According to this technique analysis of many small spots is performed to facilitate large scale nucleic acid analysis, thus enabling simultaneous analysis of thousands of DNA sequences. This technique is considered an
20 improvement on existing methods, which are largely based on gel-electrophoresis. For a review, see *Nature Gen.* (1999) 21 Suppl. 1. There are microarrays of different densities. High density microarrays usually have a density up to about 10^5 spots per cm^2 . Low density microarrays contain at least about 5 spots per cm^2 .

In order to fix discrete nucleotides on a solid support e.g. glass slides, one of
25 two approaches is normally used. The nucleotides are either synthesized or spotted on the glass slides. In the first approach, oligonucleotides are built by light-directed sequential oligonucleotide synthesis on the solid support; see U.S. Patent No. 5,925,525 and WO 97/27317. Light is used to activate a predetermined site for the chemical coupling of a nucleotide. An array of nucleotides is built in successive rounds of this site-directed light-
30 activated building process. In the latter approach, oligonucleotides are synthesized separately in an individual fashion and later bound to the solid support with NH_2 linkage. The solid support is coated, e.g. with silane or poly-L-lysine, and a robotic arrayer is used for printing. This robot arrayer may be based on different principles, such as piezoelectric printing, and the ring and pin mechanism.

The use of ligase in microarray analysis has been mentioned in WO 97/31256 and US 5,242,794 as a way for detection of one or more of a plurality of sequences differing by one or more single base changes, insertions, deletions or translocations in a plurality of target nucleotide sequences. The ligase step in the procedure is similar to the one mentioned for the Point-EXACCT procedure, except that exonuclease is not a part of the procedure. As solid support in US 5,242,794 a filter is used.

None of the procedures mentioned above use streptavidin coated glass slides as solid support for microarray analysis. The strong binding with biotin labeled substances on the streptavidin coating facilitates efficient detection, e.g. hybridization of nucleic acids under stringent conditions. Furthermore, for nucleic acid analysis in combination with high efficiency procedures for single strand preparation of double stranded amplification products, such as exonuclease, low target frequency on microarray analysis is feasible. The utility of DNA arrays for genetic analysis has been demonstrated in numerous applications including mutation detection, genotyping, physical mapping and gene expression monitoring. The basic mechanism is hybridization between arrays of nucleotides and target nucleic acid. This requires rather pure samples, typically more than 10-20% of target DNA in a mixed population before a signal is obtained. However, the presently available DNA array techniques could not be used for screening purposes where a low amount of target cells are present. (Gundersen K.L., *et al.*, *Gen. Res.* (1998) 7:1142-53).

In summary, the microarray technique allows large scale nucleic acid analysis, but require a large amount of target cells, since the detection mechanism is based on hybridization. Hence there is a need for optimization.

The present invention provides a further improvement of the point-EXACCT method using the microarray technique, wherein the benefits of both methods are successfully combined.

Summary of the invention

In accordance with the present invention there is provided a method of detecting the presence of a nucleotide sequence within a double-stranded DNA in a sample comprising the following steps:

a. coating a solid support with a first layer of biotinylated serum albumin, and a second layer of streptavidin molecules having sufficient density to perform efficient microarray analysis;

b. digesting the double-stranded DNA with an exonuclease to convert double-stranded to single stranded DNA, derived from a mixture of target cells and other cells, to a single-stranded DNA;

c. capturing a first nucleic acid probe adapted by biotin to said coated solid
5 support defined in step a.;

d. hybridizing (i) the single-stranded DNA with the first nucleic acid probe, and (ii) a second nucleic acid probe labeled with a detectable moiety which can hybridize with the single-stranded DNA adjacent the hybridized first nucleic probe;

e. ligating the hybridized first and second nucleic acid probes in case of
10 perfect match only;

f. denaturing the ligated first and second nucleic acid probes from the hybridized single-stranded DNA;

g. removing non-covalently bound labeled probes and single stranded DNA;
and

15 h. detecting captured detectable moiety indicating the presence of the nucleotide sequence within the double-stranded DNA in the sample;

characterized in that steps c.-h. are performed by microarray technique.

In one embodiment of the invention, step d (ii) is adapted with the use of a mixture of partly randomized probes to allow detection of mutations without knowing the
20 site and type of mutation beforehand.

In one aspect of the invention, the capturing step is performed by capturing the first nucleic acid probe to a solid support wherein this solid support preferably is of glass, most preferably Starfrost glass.

In a preferred embodiment of the invention, the support is homogeneously
25 coated by biotinylated serum albumin and streptavidin.

In another aspect of the invention, the first nucleic acid probe is printed on the solid support.

In still another aspect of the invention, the first nucleic acid probe is built on the solid support by light-directed oligonucleotide synthesis which is subsequently used for the
30 remaining of the point-EXACCT procedure including the ligase step.

In still a further aspect of the invention, the detectable moiety on the second nucleic acid probe is digoxigenin, and the detecting step is performed by binding the digoxigenin with anti-digoxigenin antibody fragments.

In a preferred embodiment of the invention, the method is suitable for detecting the presence of a point mutation within the double-stranded DNA, wherein the first nucleic acid probe contains a nucleotide complementary to the nucleotide of the point mutation at the 3' end and adapted with a moiety at the 5' end which can be captured to a solid support, and the second nucleic acid probe is labeled with a detectable moiety at the 3' end which can hybridize with the single-stranded DNA adjacent the hybridized first nucleic acid probe, and wherein after ligation and denaturation the presence of captured detectable moiety indicates the presence of the point mutation within the double-stranded DNA in the sample.

In another preferred embodiment of the invention, the method is suitable for detecting the presence of a point mutation within the double-stranded DNA, wherein the first nucleic acid probe contains a nucleotide at a first end complementary to the nucleotide of the wild type nucleic acid sequence at a nucleotide position suspected to be a point mutation and adapted with a moiety which can be captured to a solid support, and the second nucleic acid probe is labeled with a detectable moiety which can hybridize to the second end with the single-stranded DNA adjacent the hybridized first end of the first nucleic acid probe, and wherein the absence of captured detectable moiety indicates the presence of the point mutation within the double-stranded DNA in the sample.

In another aspect of the invention, a device is provided comprising a solid support, preferably of glass, most preferably Starfrost glass, which is suitable for carrying out the detection methods according to the present invention. Preferably, said support is coated with biotinylated serum albumin and streptavidin.

In still another aspect of the invention, a kit is provided comprising a device suitable for carrying out the detection methods according to the present invention with a solid support containing a series of first nucleic acid probes.

In yet another aspect of the invention, a kit is provided comprising a device suitable for carrying out the detection methods according to the present invention with a solid support containing a duplicate series of first nucleic acid probes with arrangement for rapid detection.

In still another aspect of the invention, a kit is provided comprising a device suitable for carrying out detection methods according to the present invention allowing serial analysis of multiple target cells on the same support.

In still another aspect of the invention, a kit is provided comprising a device suitable for carrying out the detection methods according to the present invention,

optionally an exonuclease, a first nucleic acid probe which binds to target DNA and which is adapted with a capture moiety, a second nucleic acid probe which binds to target DNA adjacent the first probe and which is labeled with a detectable moiety, optionally a ligase, a first antibody that binds to the detectable moiety of the second probe, a second antibody
5 that binds to the first antibody and is labeled with an enzyme for color reaction or labeled with a fluorochrome, and a chromogen.

In another preferred embodiment of the invention, a method is provided for organizing microarray analysis on a solid support for rapid visual detection of abnormalities which comprises arranging a duplicate set of probes where the first series of
10 arrays are for the wild-type mutation order and the second series of arrays are for the classical sequencing order.

These and other aspects of the invention will be outlined in more detail in the following description.

15

Brief description of the drawings

Figure 1 depicts a glass slide which is suitable for use as a glass support for the detection methods of the present invention. Squares were manually drawn on the slides, preferably having an area of about 0.25 cm², with a pap pen in order to keep the fluid on this square. Eight squares are drawn on one slide, four for hybridization and four
20 for ligation and denaturation.

Figure 2 depicts a possible order for duplicate nucleotide signal, allowing rapid visual analysis.

Figure 3 shows the wild type signal for consecutive series of nucleotides.

Figure 4 shows besides the wild type signal background signals from the three
25 remaining probes.

Figure 5 shows a possible signal distribution for point mutation in a sample with target fraction.

Figure 6 shows a possible signal distribution for a small deletion or insertion in a sample with high target fraction.

Figure 7 shows a possible signal distribution for a point mutation in a sample
30 with a low amount of target cells.

Figure 8 shows a possible signal distribution in case of heterozygosity.

Figure 9 shows a possible signal distribution for loss of heterozygosity for single nucleotide polymorphism in a sample with high target fraction.

Figure 10 shows a possible signal distribution for loss of heterozygosity for single nucleotide polymorphism in a sample with low fraction of target cells.

Detailed description of the invention

5 The present invention provides a significant improvement of the method of detecting the presence of a nucleotide sequence and preferably a point mutation within a double-stranded DNA in a sample, which is disclosed and claimed in U.S. Patent No. 5,744,306, the disclosure of which is incorporated herein by reference. The improvement is based to a great extent on replacing in the "Point EXACCT" detection method the
10 microtiter plates as solid supports by microarray technology which involves the use of solid e.g. glass supports.

The terms 'microarray' or 'chip' technique or technology, as used herein, are synonyms and are meant to indicate analysis of a plurality of small spots of nucleic acids distributed on a small surface area to facilitate large scale nucleic acid analysis enabling
15 the simultaneous analysis of thousands of DNA and/or RNA sequences. The terms are likewise applicable to the analysis of peptides or proteins in a similar way.

It has now surprisingly been found that the microarray technology can be successfully applied on the Point-EXACCT detection method, replacing microtiter plate format which uses a volume of about 100 μ l per well with streptavidin coated glass slides
20 using a volume of 120 μ l for all spots on the support, and thus enabling to perform Point-EXACCT with smaller samples and at much larger scale of operation.

The detection mode may vary but the invention can be suitably carried out with absorption microscopy and other modes, such as fluorescence and laser scanning microscopy, which will be evident to a person skilled in the art.

25 In order to perform the microarray technique successfully on Point-EXACCT, it has been found that excellent results are obtained with glass slides, in particular starfrost glass slides, but other materials such as silica or glass slides of other types may also give satisfactory results. This can easily be determined by a person skilled in the art. The glass slides preferably contain squares drawn on the glass in order to keep the fluid on this
30 square. The size of the squares is not critical. A preferred size has an area of about 0.25 cm^2 , so that 8 squares can be drawn on 1 glass slide, four for hybridization and four for ligation and denaturation. This is convenient for testing one base on a slide.

The slides normally are coated by conventional techniques which include coating with biotinylated serum albumin (usually BSA) which is soaked by streptavidin.

Excellent results are obtained when a first homogeneous coating of biotinylated BSA is applied which after drying is incubated by streptavidin. A high concentration of biotinylated albumin is necessary to get sufficient binding sites for the capture probes. In the concentrations used, the effect of the surface tension generally will result in an inhomogeneous protein distribution. Homogenation is obtained by additional measures before drying, for example covering the protein layer with non-adherent weight or using a surfactant. In an embodiment of the present invention the coating allows high density deposition by robots of biotin labeled substances such as nucleic acids and proteins. The coated supports can be stored for long periods of time (up to 12 months) at room temperature. The surface area of the coated support is not critical and may vary according to the wishes of the user. Usually it will be up to about 10x10 cm². Slides, preferably glass slides, and more preferably Starfrost glass slides of about 7.5x2.5 cm are convenient and give satisfactory results, and are therefore preferred. The coating procedure described above which results in a homogeneous coating layer is simple and the capture probes are linked neither chemically nor with UV radiation to the glass surface. A homogeneous layer of biotin-labeled substances such as nucleic acids and proteins can be suitably used for microarray analysis.

The array technique on the Point-EXACCT detection method according to the invention worked well with high concentrations of all products. Once the principle was established, the concentrations were brought back step by step to fmol-pmol range. Thus, the concentrations of the products to be detected and the reagents used in the modified Point-EXACCT method according to the invention usually differ from those applied in the conventional Point-EXACCT method and can be determined and optimized by routine experimentation.

As appears from the Experimental Part hereinbelow, suitable and preferred concentrations are as follows:

- for the biotinylated bovine serum albumin coating on the glass slides: about 20 µg/ml
- for the streptavidin coating on the glass slides: about 40 µg/ml
- for the biotinylated capture probes: about 5 ng/0.15 µl of biotinylated probe
- for the digoxigenin-labeled capture probe: about 5 ng/0.15 µl of digoxigenin-labeled probe
- for the amount of (PCR) product: about 1µg per square of 0.25 cm²
- for the concentration of anti-digoxigenin antibody: about 5µl with a concentration of about 1 µg/ml

- for the concentration of rabbit anti-mouse labeled with alkaline phosphatase: about 2.25×10^{-7} g/5 μ l.

It appears from these experiments that the concentrations of all products used for Point-EXACCT could be brought back to fmol-pmol range for a surface of about 5 0.25 cm². A further reduction in the surface of the capture probe area is possible and will lead to a further decline in the number and/or concentrations of these products needed for a detectable signal. The amount of molecules needed for a surface of 0.25 cm² usually is in the range of about 10^{11} - 10^{12} molecules.

In order to prove the efficiency and effectiveness of the method according to 10 the invention, a variety of tissue samples was tested with the modified Point-EXACCT method involving the array technology, including wild types and samples which were known to contain certain K-ras mutations, which were predetermined by the conventional Point-EXACCT procedure. The results of the conventional Point-EXACCT and the microarray approach were identical, the same mutations were found with both tests.

15 In another embodiment of the invention, a vertical slide holder is used comprising a small fluid-containing incubation 'chamber'. One of the broad vertical walls of the incubation chamber consists of the solid support with the microarrays (about 5x2 cm). The distance between both broad vertical walls is small, e.g. 80 microns, which is sufficient for arrays of nucleic acids, and also for individual cells and tissue sections. 20 Reagents can be added on top of the upper wedge shaped and wider part of the incubation chamber and the incubation space is filled by capillary and gravity force. Excess fluid on the upper level will flow through the incubation space by gravity. The incubation space will remain filled with fluid due to the capillary force. By subsequent variations in the contents of the fluid the whole incubation chamber with its microarrays on 25 one of the walls is rapidly changed. In this way the handling is facilitated and can be automatically performed by a pipetting robot (e.g. MARK 5). In the vertical mode the order of sample and detection probe hybridization is also changed. In horizontal mode target nucleic acids and detection probe can be hybridized together. In the vertical mode target nucleic acids are hybridized first, any excess is washed away and the detection probe is 30 then hybridized to the target nucleic acid.

Another embodiment of the invention is the parallel hybridization in the vertical mode of two solid supports with the same capture probe composition. The first support is used for quantification of the target DNA without ligation and denaturing step (also called hybridization only), whereas the second support is used for quantification after ligation,

denaturing, and staining. The ratio between the signals of the corresponding capture probe determines the outcome. This ratio is obtained by dividing the signal of the support 'without ligation and denaturation' by the signal of the support 'with ligation and denaturation'. This setup requires high quality of coating of the supports, and of the spotting steps, emphasizing the relevance of the coating according to the present invention. All other steps are performed in parallel by robotic and do not give rise to any essential variations.

While conventional microarray technology is based on the hybridization mechanism, as outlined above, the present invention of the Point-EXACCT microarray method uses beside two hybridizations the ligase reaction for signal discrimination without significant loss of signal intensity. This has the advantage that also a lower amount of target cells can be present in the biological sample. While in the conventional microarray technology from 100% to 10% of the sample material has to be hybridized in order to obtain a reliable signal discrimination, this range is extended in the method of the present invention to from 100% to about 0.1%.

As compared to the hybridization in the conventional microarray technology the subsequent ligase step according to the present invention has certain distinct advantages. Identical hybridization conditions can be used in both instances. Consequently, discrimination between matches and mismatches is very high, much higher than can be achieved with any other methods, such as oligonucleotide mutation specific hybridization wherein the effects of GC content were only somewhat neutralized in high concentrations of quarternary or tertiary amines.

Although in the microtiter based Point-EXACCT procedure the signals of the well 'without ligation and denaturation' and the signal of the well 'with ligation and denaturation' are in close approximation to each other, in the microarray format of Point-EXACCT these signals are preferably obtained by different supports. This allows the calculation of the ratio as mentioned above. An embodiment of the invention is that with the microarray format the support 'with the ligation and denaturation' steps is sufficient in itself to acquire the information about the presence of mutation. Since information about the wild type signal can be obtained from a series of contiguous nucleotide positions, a normal range with its distribution (i.e. without mutations) can be calculated for each support. With these data a subset of probes usually is sufficient for predicting the normal values of the other wild type probes. When one wild type probe is significantly different from the expected normal range, a mutation is present (see also Figure 3).

In the design for the capture probe composition there are several options. For example, for a specific base all of the four theoretical possibilities are spotted adjacent to each other. With Point-EXACCT the wild type signal should be positive after ligation. If a point mutation is present in a target nucleic acid and this target nucleic acid is present in sufficient quantities, the probe detecting the mutation will be positive as well. The extent of positivity, i.e. the amount of signal is largely dependent on the amount of target nucleic acids present. The remaining two probes are negative and serve as a negative control. However, the hybridization signal in the support that is used for 'hybridization only' should be high as well and serves as a quality control for hybridization and spotting.

Another capture probe design useful for known single nucleotide polymorphism analysis is the presence of only two capture probes on the solid support. For a single nucleotide polymorphism locus either homozygosity for one of the two possibilities, i.e. probes or heterozygosity is present. After Point-EXACCT 'with ligation and denaturation' only a high signal will be present for the homozygous nucleotide. In contrast, in case of heterozygosity both probes will show a clear signal. However, this signal amount is lower than for homozygosity. Ratio calculations provide clear distinction between homozygosity and heterozygosity. This part of the invention works satisfactory on blood samples containing white blood cells with a fairly homogeneous composition of DNA.

Another design of probe deposition according to the invention is the presence of duplicate series of capture probes with a different arrangement. In the sequence type of arrangement, the nucleotides usually are positioned in four arrays where the ends of the capture probe on the 3' site will be the same. Thus, one array will end on adenine (A nucleotide), the others on guanine (G nucleotide), thymine (T nucleotide) and cytosine (C nucleotide), respectively. When microarrays with duplicate capture probes are used, the combination of two different capture probes arrangements is useful. The second arrangement is suitably based herein on wild type option in the first arrangement and the three other nucleotide options are then deposited on the next three arrays. If no mutations are present, only the wild type array should have high signals with Point-EXACCT. Any change from normal is an indication of a mutation.

Practical examples using the latter arrangement are as follows:

(1) A point-mutation that with Point-EXACCT will result in one capture probe spot in one of the other three lanes with a high signal. In addition the ligase reaction will be hampered on the 3' site for a short number of nucleotides as described above.

(2) In case of small deletions or insertions this will lead, in addition to the mutation signal, to a longer track with reduction of wild type signal than for the point-mutation. The size of this reduction track is dependent on the size of the deletion/insertion. Alternatively, for easy recognition of single nucleotide polymorphic sites, these sites may be clustered.

The advantage of this capture probe arrangement is that any deviation from a long line of positive signals is detected with the naked eye in a split second. Thus, the analysis according to the method of this invention is very rapid. By arranging the duplicate wild type order and sequence order of probes adjacent to each other, the wild type arrangement can be used for rapid detection of the mutated region and the adjacent sequence order to determine or get an approximation of the type of mutation. According to a preferred design of the arrays, one lane is used for marking, the next four lanes are used for the wild-type arrangement of the probes, and the next four lanes for sequencing arrangement. The mark lane can also be used to depict the single nucleotide polymorphic loci. By this arrangement rapid visual analysis of 5x2 cm microscope object slides is conveniently performed within a few minutes.

In a further embodiment of the invention loss of heterozygosity is detected applying the characteristic of heterozygosity for single nucleotide polymorphism with the modified Point-EXACCT method according to the invention. Loss of an allele frequently occurs in tumor cells. If the biological sample contains a high proportion of tumor DNA, such as DNA from a tumor cell line or laser dissected tumor cells, and a part of the allele containing the single nucleotide polymorphism is lost, then only one of the two probes will be positive with Point-EXACCT. This can be detected by comparison with another sample of the patient containing wild type DNA, e.g. from a blood sample or buccal smear. In a sample containing a mixture of wild type and tumor cells, with assumptions about the loss of the allelic site in the tumor after Point-EXACCT, a normal signal is shown for the nucleotide that is not lost, but the signal of the lost allele will be lower than expected. The amount of signal is proportional to the fraction of wild type DNA. Consequently the ratio between both single nucleotide polymorphic nucleotides will also change and be different from normal. Biological samples frequently contain a mixture of cells. Typical examples are biopsy and resection specimens of tumors, sputum, liquor and urine etc.

Previously mentioned detection procedures with Point-EXACCT require a prior knowledge about the nucleic acid sequence to be analyzed for example detection of a specific point-mutation, single nucleotide polymorphism, or loss of such a polymorphism.

With the microarray Point-EXACCT technique mutations can also be detected which are not known beforehand. In malignant tumor development some genes, especially tumor suppressor genes are known for a mutation prone region. When for such a region or gene a large number of probes with for each nucleotide all possible combinations of capture
5 probes are spotted on the microarray, then with Point-EXACCT a mutation in the target DNA will be detected on the specific site by hampering of the ligase reaction for the wild type probes and thus result in a reduction of the wild type signal. This reduction will be marked the most at the first base of the mutation. On the next bases the signal will be reduced depending on the diminishing of the ligase reaction. The larger the distance
10 between the (end of the) mutated site the more nucleotides at the ligation site will be complementary to the wild type probe with a consequent increase in wild type signal.

In the previous embodiment of the invention the second oligonucleotide with detection moiety is specific for a certain genomic region.

In another embodiment of the present invention the second labeled oligo-
15 nucleotide may contain nonspecific nucleotides, such as inosine or others, in order to reduce the number of second oligo probes for a more general application. More specifically, a combination of 'partly random probes' may be useful. The so called 'partly random probes' are oligomers of about 12 or preferably less 9 or more preferably 6 nucleotides long and designed in the following way. On each position only the same 3 of
20 the 4 possible oligonucleotides are randomly incorporated. A mixture of the possible four different oligonucleotides results in a high number of possible random probes. In more detail, for example for DNA, the mixture consists of (GAT) n , (GAC) n , (GCT) n and (ACT) n , where n is the length number of the oligonucleotide, e.g. 9, 8, etc. The 3' end of these probes is labeled with digoxigenin or other labels (see below). In this way the mixture
25 works for hybridisation and detection. In case the ligase reaction is used, the 5' end of these oligomers first needs to be chemically phosphorylated. Apart from the above described composition, incorporation of one or more non-specific nucleotides at any of nucleotide positions is useful. For RNA the pyrimidine thymine (T) is replaced by uracil (U). The use of the mixture of 'partly random oligomers' in the microarray analysis instead of
30 the specific oligonucleotides, allows a wider use of applications. One option is the design with capture probes aligned with the four possible options for each DNA/RNA base, representing an array with a known part of the genome. If after the hybridisation of target sequences and detection probes, ligation and staining procedure just the wild type capture probes are visualised the normal sequence is present only. However, if (part of) the other

capture probes are visible, this denotes the presence of possible point mutation(s), deletion(s) and/or insertion(s), provided that the range of deletion(s) / insertion(s) is at least partly covered by the array of capture probes. Detecting normal sequences or aberrations in unknown regions of the genome is not part of this invention.

- 5 Summarizing, the new modified Point-EXACCT method according to the invention involving the microarray technology is fast (more than 1000 nucleotides in 200 specimen can be analyzed in one day), highly specific and highly sensitive, relatively cheap and it can be automated. The method is also user-friendly, i.e. the convenient solid open format of the microscope slide, the non-radioactive, non-toxic, low-volume
- 10 hybridization solution, and the comforting knowledge that the arrays are cheap and easily replaceable. Finally, the transfer to a smaller scale has many advantages, including working with smaller samples (fmol/pmol level), whereby lots of samples can be processed at the same time. Ten thousand probes can be spotted on one glass slide which enables the use of the modified Point-EXACCT method for large scale testing, e.g.
- 15 simultaneous testing of a plurality of sequences from one gene or different genes from one or more patients.

The modified Point-EXACCT method according to the invention is also suitable for large scale screening of point mutations, insertions, deletions, and loss of heterozygosity at the same time.

- 20 The method of the present invention involving the array technology also offers a plurality of possibilities for further improvement. For example, the biotin labeled substance can serve as capture probe for subsequent binding of nucleic acids (NA)/protein. (NA/Proteins of interest, NPOI). The NPOI in turn can be made visible by direct labeling during synthesis of NPOI before capturing or, in case of unlabeled NPOI, by subsequent
- 25 binding of labeled detection probe.

Preferably a fluorescence mode is used for reasons of higher sensitivity, larger dynamic range, instead of extinction mode. The latter method has the advantage that the outcome can be made visible with regular light microscopy.

30 General applications using the modified Point-EXACCT method of the invention

nucleic acid analysis:

- DNA point mutation detection
- DNA deletion detection, same way use of specific probe/ indifferent probe
- DNA insertion, same way use of specific probe/ indifferent probe
- 35 - DNA methylation detection

- DNA single nucleotide polymorphism detection
- RNA after cDNA conversion (labeled = i, unlabeled = ii)
- RNA direct (approach ii)

Protein analysis for presence/amount of protein

5

Specific applications

- detection of abnormal DNA/RNA in cancer specimen
- (early) detection of abnormal DNA/RNA in body fluids, such as blood, urine, sputum, bile, lavage fluids, cerebrospinal fluid, stool
- 10 - (early) detection from cancer cells in blood/bone marrow
- prediction of treatment/chemosensitivity of tumor cells
- detection of specific microorganisms e.g. for clinical and food applications, such as human papilloma virus, legionella, tuberculosis, etc.
- comparison of levels of gene expression.
- 15 - analysis of genetic predisposition for specific diseases, e.g. lung cancer, COPD, atherosclerosis.

Although the present invention is herein described in certain typical embodiments, it will be understood that variations may be made without departing from
20 the spirit of the invention. For example, the modified Point-EXACCT method according to the invention is typically described herein using alkaline phosphatase for detection with absorption microscopy. Evidently, this method can be replaced by different labels, including radio nucleotides, substrates, magnetic particles, heavy metal atoms, and particular fluorescers, chemilumescers and spectroscopic labels, other enzymes than
25 alkaline phosphatase, etc., which may change the sensitivity and/or the dynamic range of the system. With an appropriate label selected, the detection system best adapted for high resolution and high sensitivity detection may be selected. As indicated above, this may be fluorescence or chemiluminescence optical systems. Other detection systems may be adapted to the purpose, e.g. IR microscopy, atomic force microscopy, electrical
30 conductance, image plate transfer, and interference microscopy (e.g. Jamin-Lebedief). Such variations which are entirely clear to the man skilled in the art, are all encompassed within the scope of the present invention.

Similarly, although the present inventions is predominantly described as a method of detecting the presence of certain DNA sequences, it will be understood by the
35 average skilled persons in the art that the same principle can also be applied for detecting

the presence of certain RNA or aminoacid sequences, making appropriate adaptations without inventive effort. These variations are therefore also encompassed within the scope of the present invention.

The following Examples which are not to be construed as limiting the scope of the invention in any respect, further illustrate the invention.

EXPERIMENTAL PART

1. Point-EXACCT on glass slides

Squares of 0.25 cm² were drawn on the glass slides with a pap pen in order to keep the fluid in this square. In this way 8 squares were drawn on 1 glass slide, four for hybridization and four for ligation and denaturation. See Figure 1.

2. Choice of the glass material

A plurality of materials was tested. Among these were glue-coated, silane-coated, uncoated, poly-L-lysine-coated, vectabond-coated, APTS-BSA-coated, starfrost, superfrost and superfrost+ glass slides. The coating of biotinylated bovine serum albumin and streptavidin gave the best results on starfrost glass slides and, consequently, this material was selected for further experiments.

Furthermore, several variants on the coating procedure with biotinylated bovine serum albumin and streptavidin were investigated. It was found that a coating with bio-BSA dried on the glass slide and then incubated with streptavidin gives the best result. Variations in hybridisation procedures led to the conclusion that the PCR product and the digoxigenin-labeled detection probe could be separately hybridised the glass slide.

3. Protocols

The experiments that were performed are schematically given below. For each step, the range of variation is shown. The washing steps throughout the protocol, indicated by "washing", were each carried out with 1x PBS/0,05 % Tween 20.

30 a. Coating with biotinylated bovine serum albumin (bio-BSA)

- maximal concentration: stock 1 mg/ml; 5µl/square
- dried/incubation; 1 hour 37 °C or overnight room temperature
- coating only with bio-BSA without streptavidin
- range of concentrations: 1 mg/ml - 2 µg/ml

- since the high BSA concentration prevents direct spreading of the solution a regular distribution can be obtained with parafilm, or adding a minimal amount of surface tension reducing agent such as a detergent like soap. The parafilm is preferred, since this gave the highest signals. The parafilm may be covered with a small weight, e.g.
- 5 another glass slide.

b. Saturation with streptavidin

- maximal concentration: 500 µg/ml; 5µl/square
 - dried/incubation; 1 hour 37 °C or overnight room temperature
- 10 - coating only with streptavidin/mixing of bio-BSA and streptavidin
- range of concentrations: 500 µg/ml - 1 µg/ml

c. Washing

15 d. Linking of the biotinylated capture probes

- maximal concentration: 1 µg/µl; 0.15 µl/square
 - air-dried/incubated; 1 hour room temperature
 - range of concentrations: 150 ng/0.15 µl - 300 pg/0.15 µl
 - the length of the linker between biotin and nucleotides may be of variable length,
- 20 4-16 carbon atoms give satisfactory results

e. Washing

f. Hybridization of the single stranded PCR product and the digoxigenin-labeled detection

25 probe

("dig-probe")

- maximal concentration: 2.5 µl PCR product + exonuclease + 0.15 µl dig probe (1 µg/µl) + 4 µl hybridization buffer; 6.65 µl/square
 - 1 hour incubation at room temperature
- 30 - PCR product and dig-probe separately hybridized; 1 hours incubation each at room temperature (R.T.)
- range of amount of PCR product: 2.5 µl - 0.1 µl per square
 - range of concentrations dig-probe: 150 ng/0.15 µl - 300 pg/0.15µl

g. Washingh. Right side of the glass slide: ligation (+ washing, denaturation and washing)

- 1.25 mU T4 DNA ligase in 5 µl 1x ligation buffer

5 - incubation: 15 minutes at room temperature

- 3x washing

- 0.07 M NaOH: incubation 2 minutes R.T.

- 2 times 0.01 M NaOH/0.05 % Tween 20

- 1x washing

10

i. Detection with mouse-anti-digoxigenin antibody ("M A-D")

- maximal concentration: 5 µg/ml; 5 µl/square

- incubation: 1 hour R.T.

- range of concentrations: 5 µg/ml - 200 ng/ml

15

j. 3x Washingk. Further detection with rabbit-anti-mouse labeled with alkaline phosphatase

- maximal concentration: 0.09 g/l; 5 µl/square

20 - incubation: 45 minutes R.T.

- range of concentrations: 90 mg/l - 1.8 mg/l

l. 3x Washing25 m. Color development

- alkaline phosphatase-substrate-1kit; incubation: 15 minutes R.T.

n. 3x Washing30 o. Covered with immu-mount

- refractory index 1.5

4. **Point-EXACCT in vertical mode on starfrost glass slides with spotted biotinylated probes**

The purpose of this procedure was to perform Point-EXACCT in a vertical mode with a pipetting robot (e.g. Mark 5) Such a robot can be used for automatic immunostainings on histologic and cytologic specimens. In addition, the biotinylated probes were spotted with a microarray robot on the coated glass slides. The protocols are
5 schematically given below.

- a. Coating with biotinylated bovine serum albumin (bio-BSA)
- concentration: 20 ng/ μ l; 100 μ l/glass slide
- dried: 1 hour 37 °C/overnight R.T.
- 5 b. Saturation with streptavidin
- concentration: 40 ng/ μ l; 100 μ l/glass slide
- incubation: 1 hour 37 °C/overnight R.T.
- c. Washing
- 10 d. Spotting of the biotinylated capture probes
- range of concentrations: 500 ng/ μ l; 100 ng/ μ l; 50 ng/ μ l; 33 ng/ μ l; 10 ng/ μ l; 3 ng/ μ l
- Genetic MicroSystems (GMS) 417 arrayer: 5 nl/spot
- section of spots: 150 μ m
15 - distance between two spots: 300 μ m
- e. Washing
- f. Hybridization of the PCR product
20 - glass slides in vertical holder: Mark 5
- 22.5 μ l PCR product; 60 μ l/glass slide
- incubation: 1 hour R.T.
- g. 3x Washing
- 25 h. Hybridization of the dig-probe
- 1.35 μ l dig-probe (33 ng/ μ l); 60 μ l/glass slide
- incubation: 1 hour R.T.
- 30 i. 3x Washing
- j. One of the two glass slides: ligation (+ washing, denaturation and washing)
- 15 mU T4 DNA ligase in 60 μ l 1 x ligation buffer
- incubation: 15 minutes at room temperature

- 3x washing
- 0.07 M NaOH: incubation 2 minutes R.T.
- 2 times with 0.01 M NaOH/0.05 % Tween 20
- 1x washing

5

k. Detection with mouse-anti-digoxigenin antibody (M A-D)

- concentration: 1ng/ μ l; 60 μ l/glass slide
- incubation: 1 hour R.T.

10 l. 3x Washing

m. Further detection with rabbit-anti-mouse labeled with alkaline phosphatase

- concentration: 0.9×10^{-6} g/l; 60 μ l/glass slide
- incubation: 45 minutes R.T.

15

n. 3x Washing

o. Color development

- alkaline phosphatase-substrate-1kit; incubation: 15 minutes R.T.

20

p. 3x Washing

q. Covered with immu-mount

- Refractory index 1.5

25

Mark 5 needs 120 μ l to fill the vertical holder, from which 40 μ l is needed to rinse the previous liquid and the remaining part to fill the incubation space with liquid. As compared to the original procedure, only half of the volume was needed, since through the capillary activity, the 60 μ l product stays in the upper half of the holder where the probes

30 are spotted.

This technique was also applied for the validation of the array approach. To this end, biotinylated probes for base 2 of codon 12 of the K-ras gene are spotted in a concentration of 100 ng/ μ l. The results were analyzed by image analysis with light microscopy.

5. Use of maximum concentrations

The use of maximum concentrations of all products gave a clear red color signal. The four hybridization signals gave a distinct signal. From the four nucleotides that were ligated and denatured, only the G-base gave a red signal. This was expected because the PCR product was wild type H716 cell line DNA. The remaining 3 bases at this side of the glass slide gave no visible signal. This experiment was repeated a few times with the same result. All settings were on maximal concentrations for the various components.

10 6. Determination of minimum concentrations of the various reagents

6.1 Variation of the concentration biotinylated bovine serum albumin

In this example, the concentration biotinylated bovine serum albumin from 1 mg/ml to 2 µg/ml was varied. The rest of the settings remained standard according to the maximum protocol.

Table 1 shows the results for the hybridization and ligation signals. A semi-quantitative score of the staining is provided.

Table 1: Results variation of concentration biotinylated bovine serum albumin

Conc. µg/ml	Hybridization signals				Ligation signals			
	G	A	T	C	G	A	T	C
1000	+++	+++	+++	+++	+++	-	-	-
400	+++	+++	+++	+++	+++	-	-	-
200	+++	+++	+++	+++	+++	-	-	-
80	+++	+++	+++	+++	+++	-	-	-
40	+++	+++	+++	+++	+++	-	-	-
16	+++	+++	+++	+++	+++	-	-	-
8	+++	+++	+++	+++	+	-	-	-
3.2	++	+++	++	++	+	-	-	-
2	+	++	+	++	+/-	-	-	-

Legend: +++ very strong staining

5 ++ strong staining

 + moderate staining

 +/- background staining

 - no staining

10 The hybridization signals gave a strong staining up to a concentration of 2 µg/ml. The staining of the ligation signal of the G-base decreased after a concentration of about 16 µg/ml. Therefore a concentration of 20 µg/ml was applied in further experiments. From this concentration, 5 µl was used for a square of 0.25 cm². Thus, for this square 100 ng biotinylated bovine serum albumin was used which corresponds with 8.8×10^{11} molecules/square or 1.46×10^{-12} mol.

6.2 Variation of the concentration of other components

20 In this example, the streptavidin concentration was varied in a range from 500 µg/ml to 1 µg/ml. For biotinylated bovine serum albumin the optimum concentration of 20 ng/µl. was used. The rest of the settings remained standard according to the maximum protocol.

The optimal streptavidin concentration appeared to be 40 ng/ μ l. This was used in subsequent experiments. For several other variations a similar setup was used and optimal concentration for concentration biotinylated probes appeared to be 5 ng/0.15 μ l will be used. This equals approximately 3.9×10^{11} molecules in a spot of 150 micron diameter.

The optimal concentration of the dig-labeled probes appeared to be of 5 ng/0.15 μ l. This 5 ng contained the same amount of molecules and mol as the biotinylated capture probes.

The minimum amount of PCR product was 1 μ g was needed for a square of 0.25 cm². This corresponds to approximately 10^{10-14} copies.

The optimal concentration of the first mouse-anti-digoxigenin antibody was 1 μ g/ml. This corresponded to 2×10^{11} molecules and 3.3×10^{-13} mol per square.

The optimal concentration of rabbit anti-mouse labeled with alkaline phosphatase was acquired with 45 mg/l RAM-AP. This equals 2.25×10^{-7} g/5 μ l used, which means 9×10^{12} molecules or 1.5×10^{-11} mol. All experiments were carried out with a horizontally positioned solid support and hybridization and washing steps were performed by adding drops and sucking it off individually.

7. Validation of the microarray approach

Tumor tissue samples with K-ras mutations (codon 12 base 2), determined with the conventional Point-EXACCT procedure, were tested with the array method. Cases with and without mutations were used. Comparison of the results of the conventional Point-EXACCT and the array approach showed a similar outcome. The same mutations were found with the two techniques.

The present disclosure is to be considered as in all respects illustrative and not restrictive, the scope of the invention being indicated by the appended claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

Revised Claims
(2 November 2001)

1. A method of detecting the presence of a nucleotide sequence within a
5 double-stranded DNA in a sample comprising the following steps:

- a. coating a solid glass support with a first layer of biotinylated serum albumin in an amount to create sufficient binding sites for the capture probes, drying said first layer and incubating said first dried layer with a second layer of streptavidin having sufficient density to perform efficient microarray analysis;
 - 10 b. digesting the double-stranded DNA with an exonuclease to convert double-stranded to single stranded DNA, derived from a mixture of target cells and other cells, to a single-stranded DNA;
 - c. capturing a first nucleic acid probe adapted by biotin to said coated solid support defined in step a.;
 - 15 d. hybridizing (i) the single-stranded DNA with the first nucleic acid probe, and (ii) a second nucleic acid probe labeled with a detectable moiety which can hybridize with the single-stranded DNA adjacent the hybridized first nucleic probe;
 - e. ligating the hybridized first and second nucleic acid probes in case of perfect match only;
 - 20 f. denaturing the ligated first and second nucleic acid probes from the hybridized single-stranded DNA;
 - g. removing non-covalently bound labeled probes and single stranded DNA; and
 - h. detecting captured detectable moiety indicating the presence of the
25 nucleotide sequence within the double-stranded DNA in the sample;
- characterized in that steps c.-h. are performed by microarray technique.

2. The method of claim 1, wherein step d (ii) is adapted with the use of a mixture of partly randomized probes to allow detection of mutations without knowing the
30 site and type of mutation beforehand.

3. The method according to claim 1 or claim 2, wherein said solid glass support is made of Starfrost glass.

4. The method of any one of claims 1 to 3, wherein first nucleic acid probes are printed on said solid glass support or are built on said solid glass support by light-directed oligonucleotide synthesis.

5. The method of any one of claims 1 to 4, wherein the detectable moiety on the second nucleic acid probe is digoxigenin, and the detecting step is performed by binding the digoxigenin with anti-digoxigenin antibody fragments.

6. A device suitable for carrying out the detection method as claimed in any one of the preceding claims, which comprises a solid glass support having a coating which is obtainable by the method comprising coating said solid glass support with a first layer of biotinylated serum albumin in an amount to create sufficient binding sites for the capture probes, drying said first layer, and incubating said first dried layer with a second layer of streptavidin having sufficient density to perform efficient microarray analysis.

7. The device according to claim 6, wherein the said solid glass support is made of Starfrost glass.

8. A kit comprising:

- a. a device suitable for carrying out the detection method according to the present invention as claimed in any one of claims 1 to 7;
- b. optionally an exonuclease;
- c. a first nucleic acid probe which binds to target DNA and which is adapted with a capture moiety;
- d. a second nucleic acid probe which binds to target DNA adjacent the first probe and which is labeled with a detectable moiety; and
- e. optionally a ligase.

9. A method for organizing microarray analysis on a solid glass support for rapid visual detection of abnormalities which comprises arranging a duplicate set of probes where the first series of arrays are for the wild-type mutation order and the second series of arrays are for the classical sequencing order.

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10. The method of claim 1, wherein prior to the drying in step a parafilm, preferably covered by a weight, or a surfactant is added to enhance the distribution of said first layer.

5 11. The device of claim 6, wherein the solid glass support is obtainable by the method in which prior to the drying in step a parafilm, preferably covered by a weight, or a surfactant is added to enhance the distribution of said first layer.

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(54) Title: IMPROVED METHOD FOR NUCLEOTIDE DETECTION AND DEVICES USED THEREIN

(57) Abstract: The present invention provides an improved method of detecting the presence of a nucleotide sequence within a double-stranded DNA in a sample comprising the following steps: a) coating a solid support with a first layer of biotinylated serum albumin, and a second layer of streptavidin having sufficient density to perform efficient microarray analysis; b) digesting the double-stranded DNA with an exonuclease to convert double-stranded to single stranded DNA, derived from a mixture of target cells and other cells, to a single-stranded DNA; c) capturing a first nucleic acid probe adapted by biotin to said coated solid support defined in step a; d) hybridizing (i) the single-stranded DNA with the first nucleic acid probe, and (ii) a second nucleic acid probe labeled with a detectable moiety which can hybridize with the single-stranded DNA adjacent the hybridized first nucleic probe; e) ligating the hybridized first and second nucleic acid probes in case of perfect match only; f) denaturing the ligated first and second nucleic acid probes from the hybridized single-stranded DNA; g) removing non-covalently bound labeled probes and single stranded DNA; and h) detecting captured detectable moiety indicating the presence of the nucleotide sequence within the double-stranded DNA in the sample; wherein steps c.-h. are performed by microarray technique. Also provided is a device and a kit suitable for carrying out said detection method.

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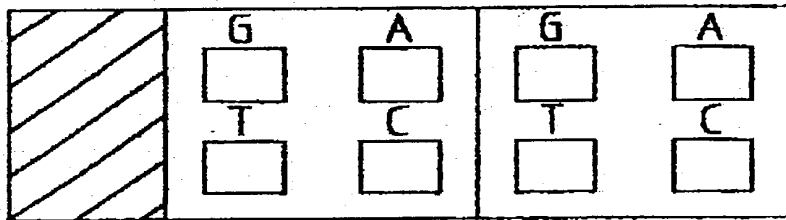


Figure 1

1 array marker	2 wild type	3 other*1	4 other	5 other	6 G	7 A	8 T	9 C
-	g	a	t	c	g	a	t	c
-	a	g	t	c	g	a	t	c
-	t	g	a	c	g	a	t	c
-	c	g	a	t	g	a	t	c
-	g	a	t	c	g	a	t	c
-	g	a	t	c	g	a	t	c
-	c	g	a	t	g	a	t	c
-	c	g	a	t	g	a	t	c
-	t	g	a	c	g	a	t	c
-	a	g	t	c	g	a	t	c
-	a	g	t	c	g	a	t	c
yes	g	t	a	c	g	a	t	c
-	c	g	a	t	g	a	t	c
-	g	a	t	c	g	a	t	c
-	t	g	a	c	g	a	t	c
-	a	g	t	c	g	a	t	c
-	t	g	a	c	g	a	t	c
-	c	g	a	c	g	a	t	c
-	c	g	a	c	g	a	t	c
	etc.							

Only bold letters give signal

*1 = possible single nucleotide polymorphism

*2 = point mutation

*3 = heterozygosity for single nucleotide polymorphism

Figure 2

Micro-array

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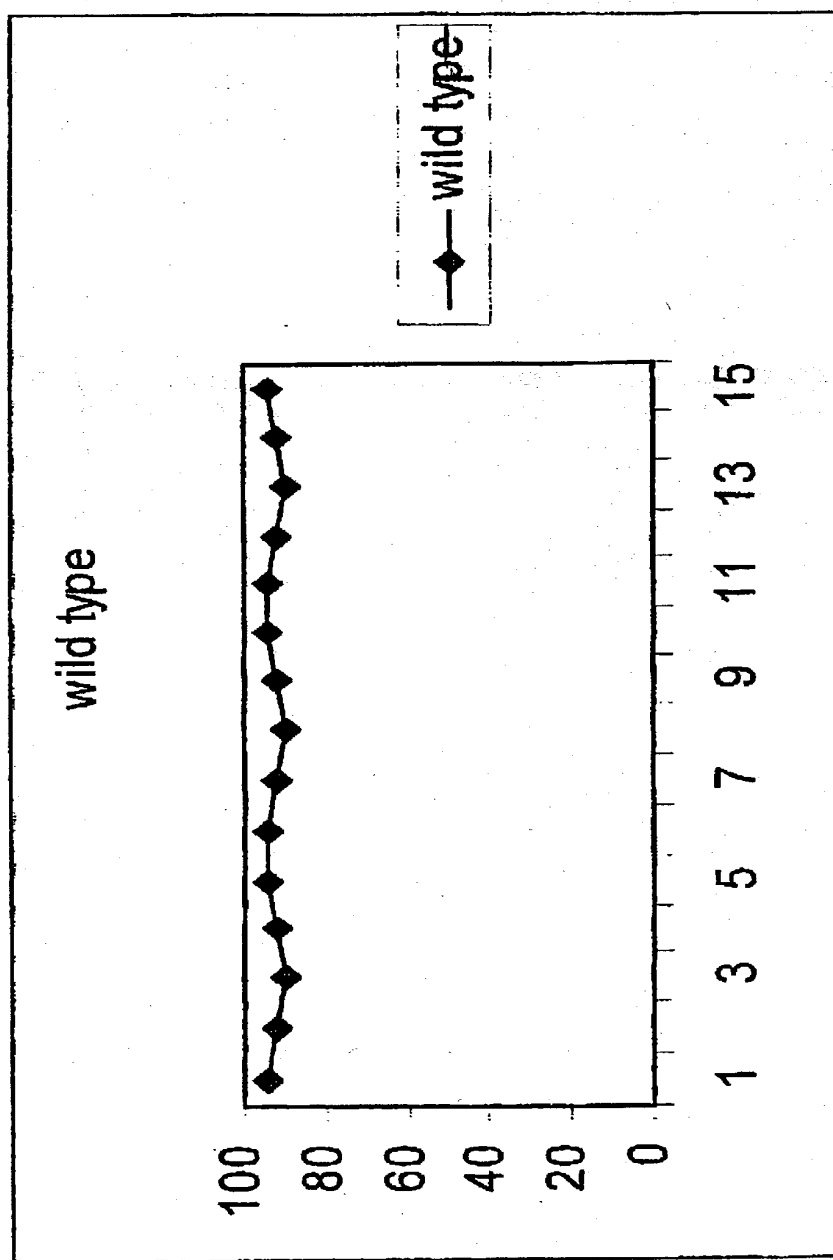


Figure 3

Micro-array

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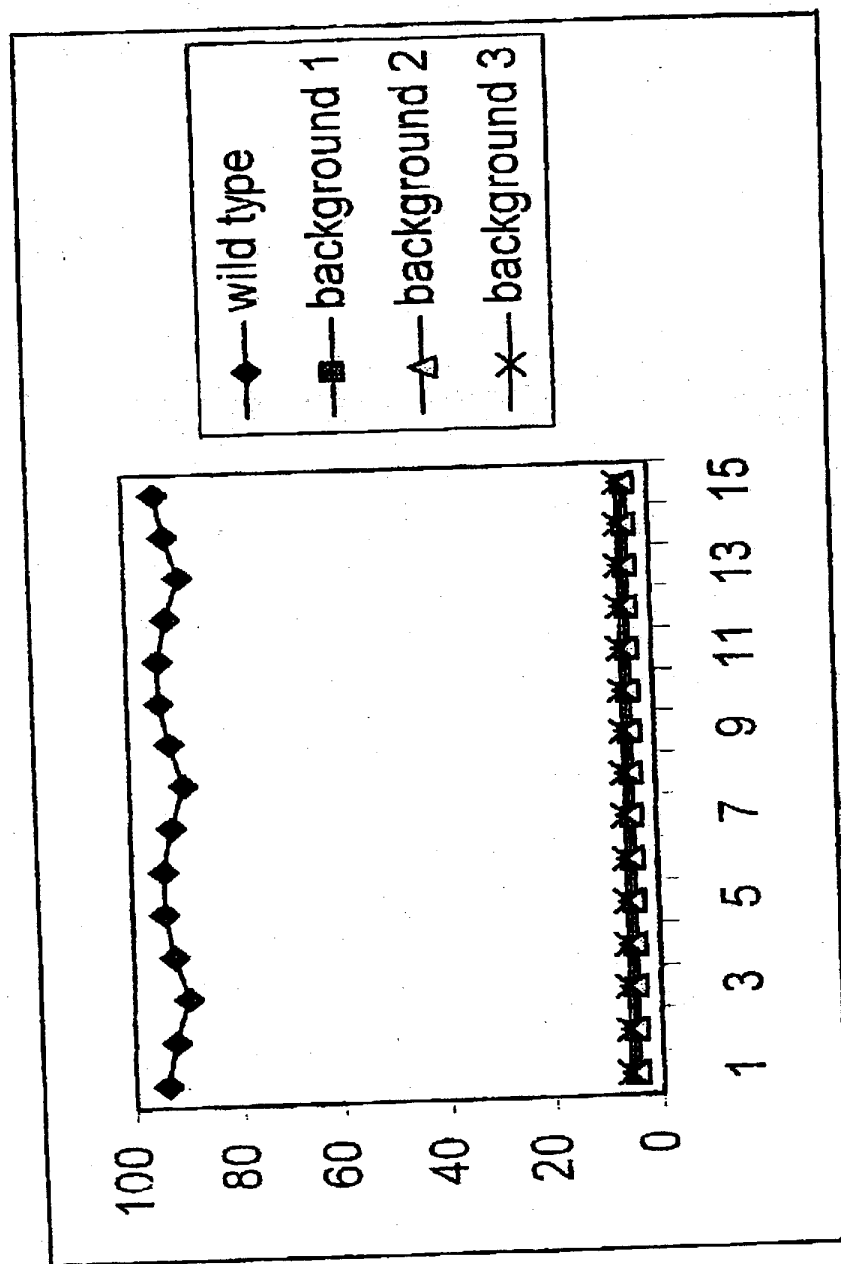


Figure 4

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Homozygous point mutation

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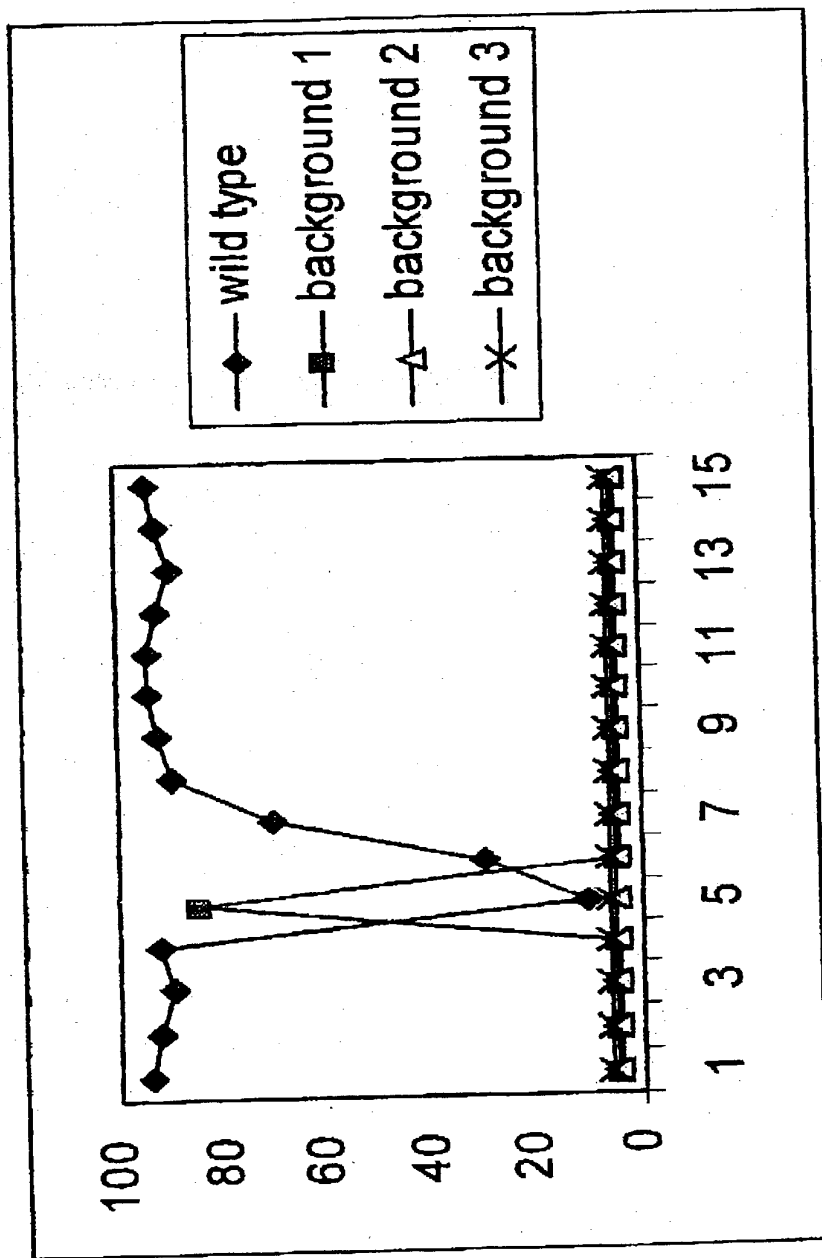


Figure 5

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Micro-array small deletion/insertion

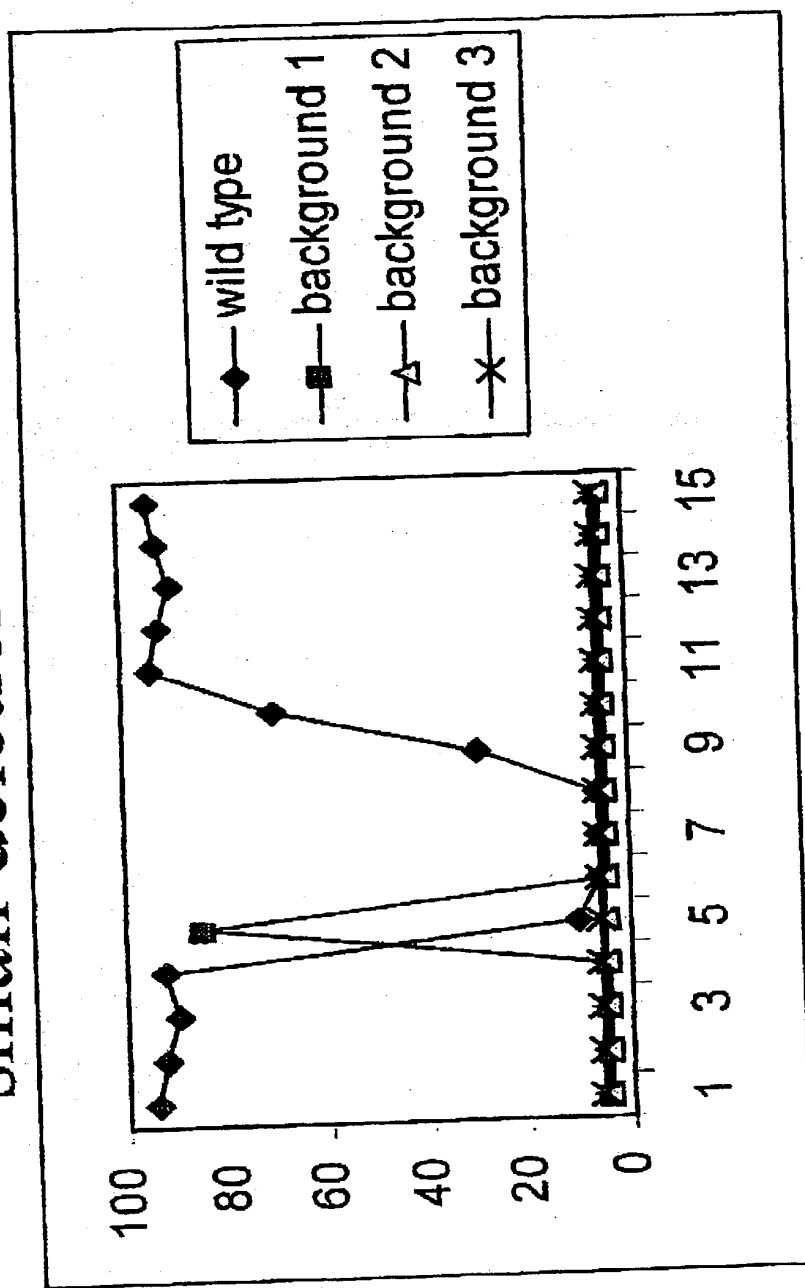


Figure 6

Micro-array point-mutation in mixture

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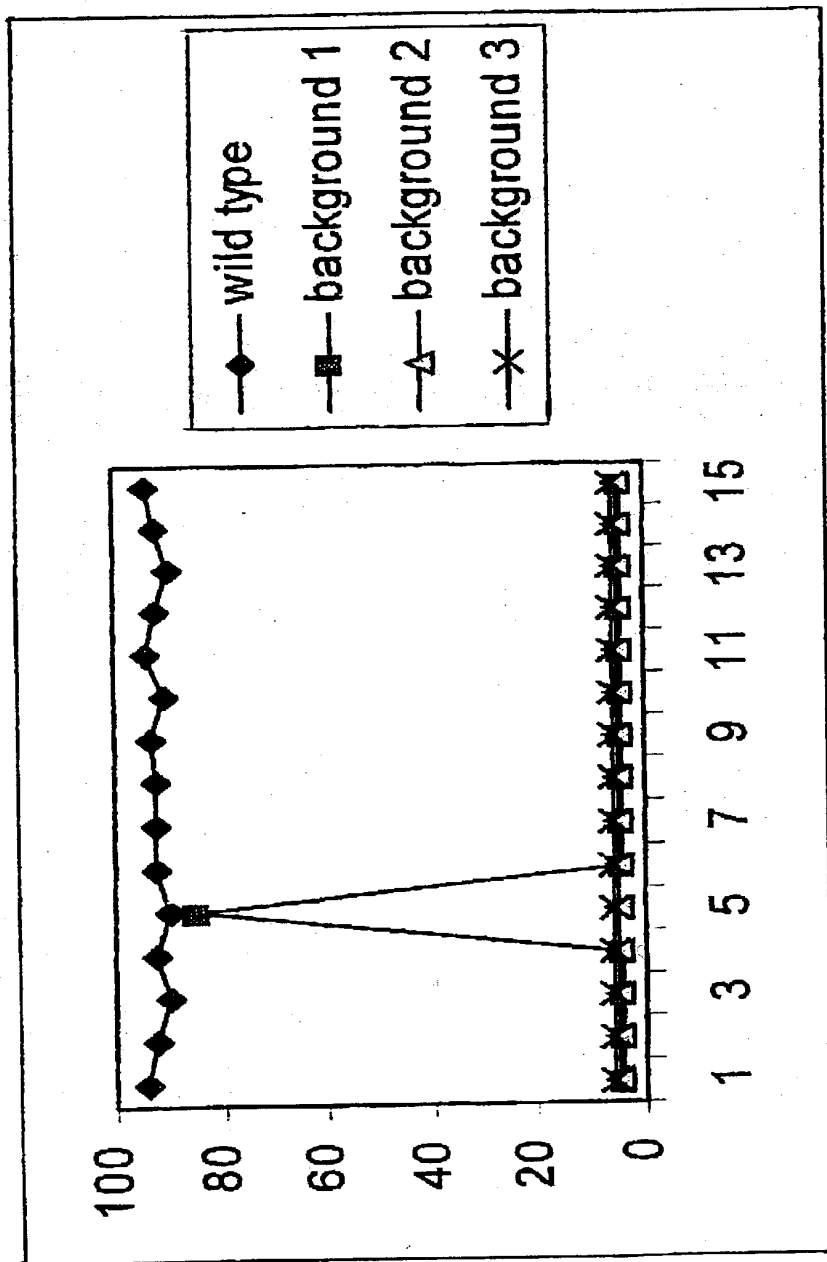


Figure 7

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Micro-array heterozygosity

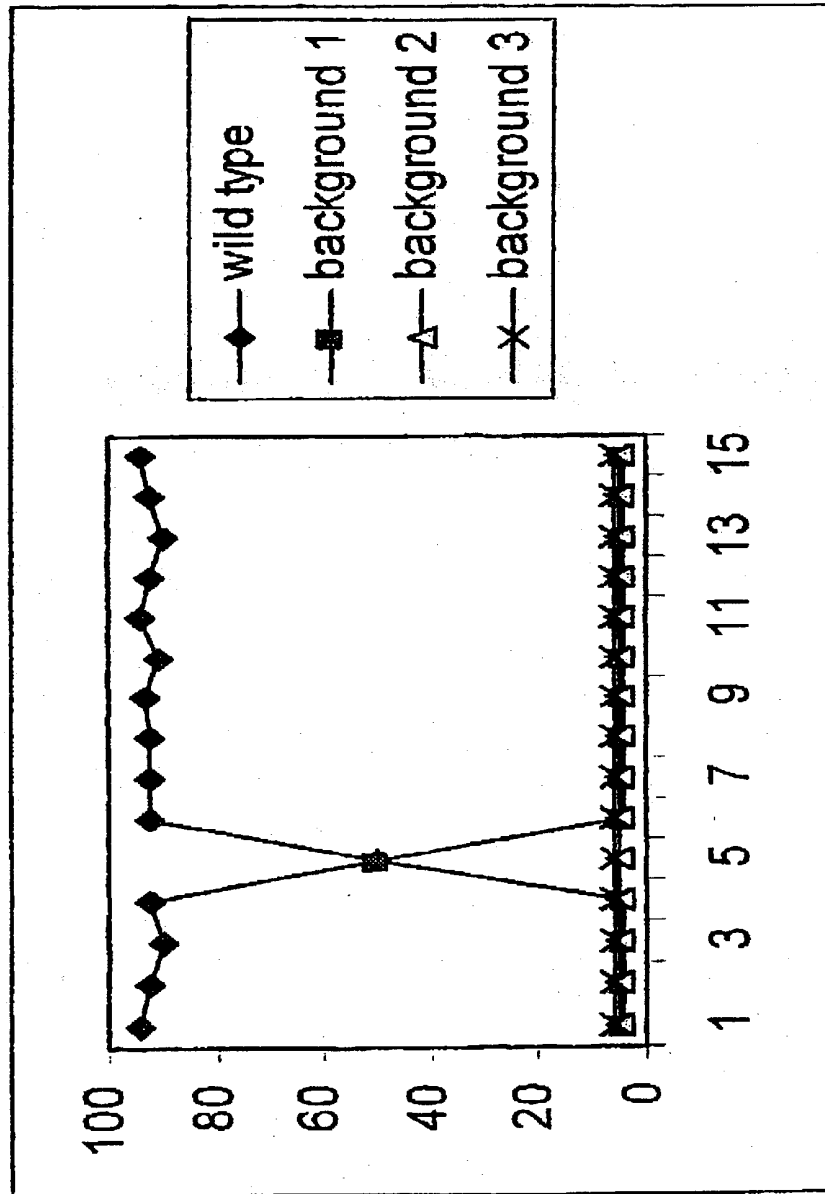


Figure 8

Micro-array LOHP pure

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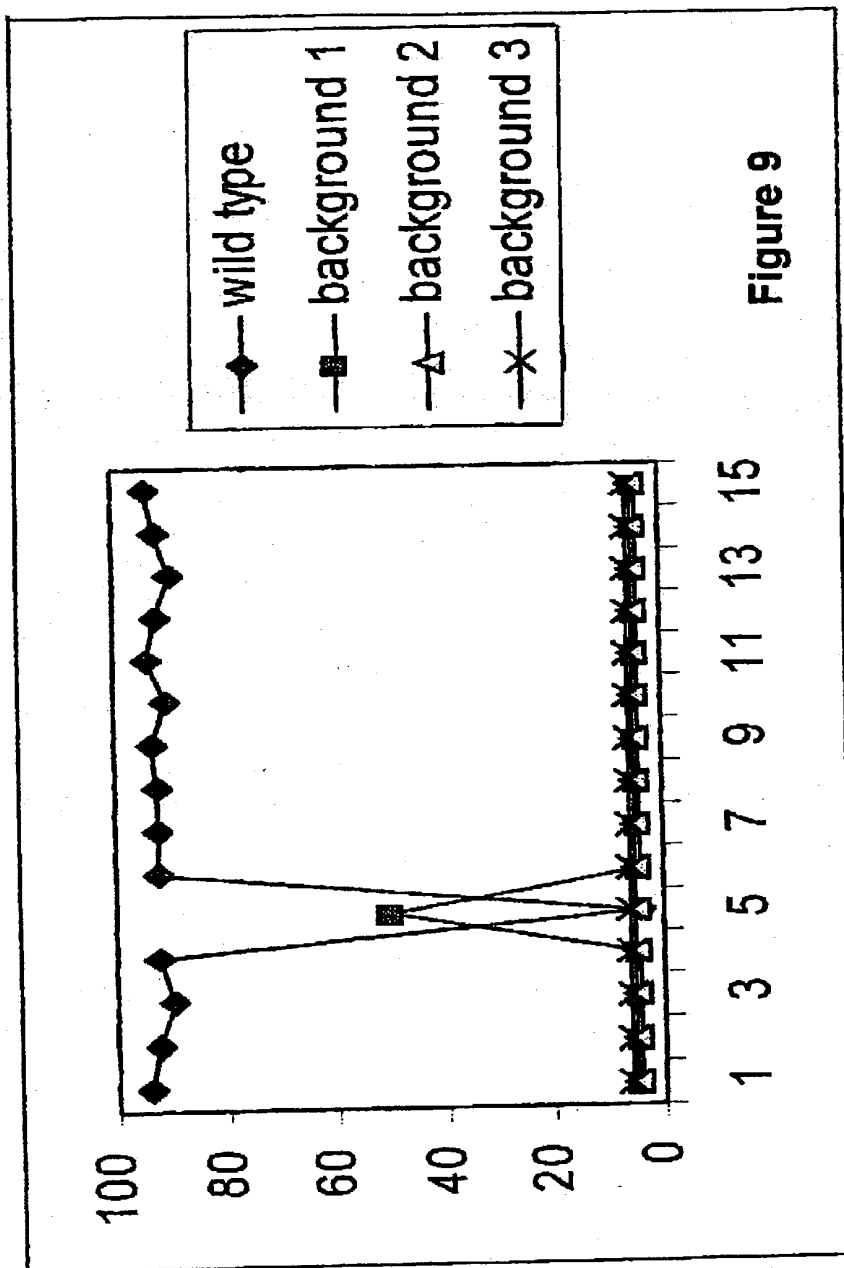


Figure 9

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Micro-array LOHP in mixture

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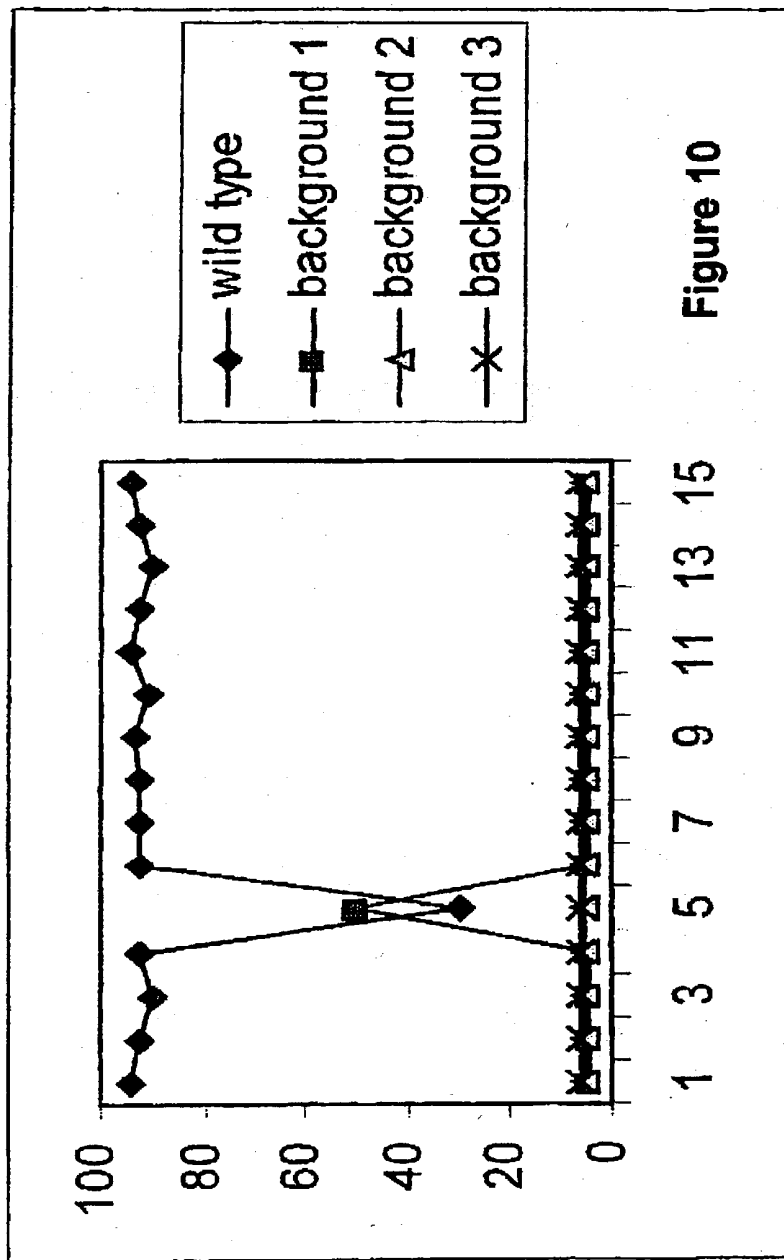


Figure 10

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DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter, which is claimed and for which a patent is sought on the invention entitled:

Improved Method for Nucleotide Detection and Devices used Therein

the specification of which
(check one)

☐ is attached hereto

☒ was filed on February 21, 2002 as

Application Serial No. 10/069,689

and was amended on _____ (if applicable)

and

_____ described and claimed in PCT International Application No. _____ filed on

and was amended on _____ under PCT Article 19 (if applicable),

(the undersigned hereby authorizes its attorney to amend this document to insert the filing date and application serial number when they become known.)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above.

I acknowledge the duty to disclose information of which I am aware which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code Section 119(a)-(d) or Section 365 of any foreign application(s) for patent, inventor=s certificate, or any PCT international application(s) which designated at least one country other than the United States of America, identified below and have also identified below any foreign application(s) for patent, inventor=s certificate, or any PCT international application designating at least one country other than the United States of America, having a filing date before that of the application(s) on which priority is claimed:

Docket No. 13189

Prior Foreign Application(s)

Priority Claimed

<u>Number</u>	<u>Country (or PCT)</u>	<u>Filing Date</u>	<u>Yes</u>	<u>No</u>
<u>99202714.4</u>	<u>EP</u>	<u>22 August 1999</u>	<u>X</u>	<u> </u>
<u> </u>	<u> </u>	<u> </u>	<u> </u>	<u> </u>

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below:

<u>Provisional Application No.</u>	<u>Filing Date</u>
<u> </u>	<u> </u>
<u> </u>	<u> </u>

I hereby claim the benefit under Title 35, United States Code, Section 120, of any United States application(s), or any PCT international application(s) designating the United States of America, that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose information which is material to patentability, as defined in 37 CFR ' 1.56, which became available between the filing date of the prior application and the national or PCT International filing date of this application.

<u>Application Serial No.</u>	<u>Filing Date</u>	<u>Status</u> (Patented, pending, or abandoned)
<u>PCT/EP/00/08270</u>	<u>22 August 2000</u>	<u>Pending</u>
<u> </u>	<u> </u>	<u> </u>

Docket No. 13189

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Please address all communications, and direct all telephone calls, regarding this application to

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

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